UNIVERSIDADE FEDERAL DA PARAÍBA

FABRÍCIO RAUAN GARCIA FURNI

WHOLE MITOCHONDRIAL GENOME CHARACTERIZATION OF THE BRAZILIAN ANTILLEAN MANATEE (*Trichechus manatus manatus*): EVIDENCE FOR A NEW BIOLOGICAL UNIT

JOÃO PESSOA
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Orientador: Prof. Dr. Pedro Cordeiro Estrela de Andrade Pinto

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ABSTRACT

Biodiversity is in constant threat due to rapid changes caused by anthropogenic impact in the world landscape. In this scenario, understanding the taxonomic composition is essential to promote appropriate delimitation of species to be evaluated for their extinction risk and protection. Animals of the *big fauna* are prone to taxonomic problems, due to the lack of materials in scientific collections and sampling difficulties. Large mammals are included, where many groups still carry many uncertainties, such as the West Indian manatees. Recent studies have shown a sum of genetic, morphological, and chromosomal evidence that suggests a new biological unit for the Brazilian Antillean manatee population, possibly a new species. However, a genomic approach is needed to provide more data about these recent changes in the family Trichechidae. This study aimed to sequence and characterizing the first whole mitochondrial genome for the Brazilian Antillean manatee (*Trichechus manatus manatus*), with intra and interspecific comparisons. Blood samples were collected from released and captive animals of northeast Brazil. DNA extraction, a novel set of primers for mitogenome isolation of Sirenians, and Next Generation Sequencing were carried out to obtain sequences from 24 samples. Bioinformatics was used to analysis of quality control, assembling, intra and interspecific genetic distances, and to define genetic profiles. A total of 3,859,650 raw sequencing reads were generated, where 18 mitogenomes were obtained with a mean coverage of 150x. The mitogenome presented 16882 bp length with 13 protein-coding genes, 18 tRNAs, 2 sRNAs, and one control region. Eight different genetic profiles were found. The distance between Antillean manatees from Brazil and manatees from Florida was ten folded larger than between manatees from Florida and *T. inunguis*. Provided there are no sampled mislabeling of hybrids, the mitogenomic intraspecific distances bring enough evidence to suggest the elevation of the taxonomic level of the Brazilian Antillean manatee population to a new biological unit. Also, interspecific findings bring to a necessity of a taxonomic review, not just for the West Indian manatee, but for the genus *Trichechus*.

Key-words: Sirenians; Mitogenome; NGS; Brazilian Antillean manatee; Taxonomy

RESUMO

Animais da *big fauna* enfrentam diversos problemas taxonômicos devido a fatores como a falta de materiais em coleções científicas ou até dificuldades em amostragens. Os grandes mamíferos estão inclusos, onde muitos grupos ainda possuem incertezas taxonômicas, como os peixes-boi das Índias Ocidentais. Estudos recentes trazem diferenças genéticas, morfológicas e cromossomais que indicam uma possível nova unidade biológica para a população brasileira de peixes-boi marinhos, como uma nova espécie. Porém, uma abordagem genômica é necessária para um melhor espectro das distâncias genéticas e estruturação das populações destes animais. Neste sentido, este estudo buscou sequenciar e caracterizar o primeiro genoma mitocondrial para o peixe-boi marinho do Brasil (*Trichechus manatus manatus),* com comparações intra e interespecíficas. Amostras de sangue foram coletadas de animais de vida livre e em cativeiro no Nordeste do Brasil. Extração de DNA, uma nova técnica de isolamento do genoma mitocondrial para Sirênios e Sequenciamento de Nova Geração (NGS) foram realizadas para obtenção de sequências para 24 amostras. Processos bioinformáticos foram utilizados para análises de controle de qualidade, montagem e comparações intra e interespecíficas. Ao todo foram 3.859.650 sequências cruas geradas, no qual 18 mitogenomas completos com uma cobertura média de 150x foram obtidos. O mitogenoma é composto por 13 genes codificadores de proteína, 18 tRNAS, 2 sRNAs e uma região controle, somando um tamanho total de 16882 pb. Análises de distância trazem uma divergência maior entre os peixes-boi marinho do Brasil e da Flórida, do que entre os animais Amazônicos e da Flórida. Levando em consideração correta identificação taxonômica das amostras, os resultados sugerem uma elevação do nível taxonômico da população de peixes-boi marinhos brasileira para uma nova espécie. Ainda, sugerem uma necessidade de revisão taxonômica, não só dentro de *T. manatus*, mas em todo o gênero *Trichechus*.

Palavras-chave: Sirênios; Mitogenoma; Peixe-boi marinho do Brasil; Taxonomia

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LIST OF ABBREVIATIONS AND ACRONYMS

AQUASIS	Associação de Pesquisa e Conservação de Ecossistemas Aquáticos
Cytb	Cytochrome Oxidase B
ESU	Evolutionary Significant Unity
FMA	Fundação Mamíferos Aquáticos
gDNA	Genomic DNA
HTS	High Throughput Sequencing
ICMBio	Instituto Chico Mendes de Biodiversidade
INCA	Instituto Nacional do Câncer
ML	Maximum Likelihood
MPEG	Museu Paraense Emílio Goeldi
mtDNA	Mitochondrial DNA
NCBI	National Center of Biotechnology Information
nDNA	Nuclear DNA
NGS	Next Generation Sequencing
PCGs	Protein Coding Genes
PCR	Polymerase Chain Reaction
RADSeq	Restriction Site Associated Sequencing
RAG-1	Recombination Activating Gene 1
SBS	Second Generation Sequencing
U.S	United States of America
vWF	von Willebrand Factor

SUMMARY

1.0 INTRODUCTION

Changes in world landscapes in the last centuries have been affecting drastically biodiversity. Every year, species are becoming extinct even before they have been described. The big fauna is one of the most endangered groups in the anthropogenic scenario. Many factors act as threats to those animals, and a good understanding of their taxonomic composition and limits is essential to promote effective conservation strategies (MACE, 2004).

Large mammals are a special group of the big fauna. Human activities, loss of habitats, hunting, and many other threats have been reducing populations and extinguished large mammals at record speeds, which place the importance of accurate taxonomic identification (CARDILLO et al., 2005). Defining biological units for large mammals is essential to promote good conservation strategies, since most of those animals work as flagship species with high trophic and ecological importance. Although, taxonomists often pose a problem working with them, where very few materials are available in scientific collections, hampering their comparison with types. Thus, many groups have unresolved taxonomic questions, which leads to an unprotected part of the big fauna.

Genetics has been an alternative to access the taxonomic complexity of biodiversity. In the last decades with the advance of sequencing technologies, species have been described or revised through molecular methods, especially large mammals, such as new species of giraffes (FENNESSY et al., 2016) killer whales (MORIN et al., 2010), river dolphin (HRBEK et al., 2014), tapirs (COZZUOL et al., 2013), and others. Even with numbers increasing for genetic species delimitation studies, many large mammals groups still carry taxonomic unresolved questions. Within them are the Trichechidae Sirenians, where the difficulty of sampling and the absence of multilocus genomic data leads to the currently uncertain composition of biological units in this taxa.

1.1 Order Sirenia

Sirenians (Order Sirenia) are strictly aquatic mammals that inhabit tropical shallow salt and fresh waters of Indian, Pacific, and Atlantic oceans, rivers, lakes, and estuaries, as well as the Amazon River Basin. Currently, the closest related groups of Sirenians are elephants (Proboscidea) and hyraxes (Hyracoidea) together they form one group called Paenungulata (MEREDITH et al., 2011). The order Sirenia is composed of only four living species within two families: Trichechidae, known as manatees, and Dugongidae, the dugong. Fossil records and molecular studies suggest a late Paleocene origin of exclusively aquatic sirenians, with diversification in the middle Eocene, with the first Dugongidae (DOMNING, 2001; BENOIT et al. 2013).

Manatees seem to have an origin from early dugongs or Protosirenides in the Eocene. However, most of the origin of the Trichechidae is based on the fossil record of the subfamily Miosireninae from the late Eocene early Oligocene (DOMNING & GINGERICH, 1994; DOMNING, 2018). The greatest diversity of sirenians occurred in the Miocene, but only the two extant families survived after the Pliocene.

The Dugongidae family has only one extant species, the dugong (*Dugong dugon* Muer 1776). This animal is restricted to the marine waters of the Pacific and Indian Oceans (REYNOLDS III & ODEL, 1991). In the 18th century, one of the two living species of Dugongidae, the Steller's sea cow (*Hydrodamalis gigas* Zimmerman 1780) was discovered in the Bering Sea. However, 27 years after its discovery, it was driven to extinction by overhunting. The Trichechidae has three recognized living species in one genus: *Trichechus*.

1.2 Family Trichechidae

The family Trichechidae emerged during the Plio-Pleistocene transition (DOMNING, 1982), where the fossil records suggest an origin in Colombia, also supported by the diversity of

mtDNA haplotypes (VIANNA et al., 2006). Both marine and freshwater lineages seem to have arisen from marine animals, but the pattern of dispersion still unclear, and a taxonomic review is needed.

The African manatee, *Trichechus senegalensis* Link, 1795 is found in shallow, estuarine, river and lagoon waters of the African West Coast (PERRINE & RIPPLE, 2002). This animal presents a thick grey to brown color, three to four nails on the pectoral fins, sparse hair throughout its body, and a paddle-like tail. The African manatee is smaller than the West Indian manatee. Observations suggest them to be partly omnivores, eating small mollusks and fish, in addition to their herbivorous diet (REYNOLDS III et al., 2018). This species is likely closely related to the West Indian manatees, originating from South American Trichechids during the Pliocene (DOMNING, 1982; DOMNING, 2018). Few studies have been addressed to the African manatee understanding, and little is known about those animals. Currently, this manatee is in danger of extinction and categorized as vulnerable by the IUCN Red List (2019).

The Amazonian manatee, *Trichechus inunguis* Natterer, 1883 is the smallest of the sirenian species, and is restricted to the Amazon river basin in South America, with a parapatric occurrence on the Amazon river mouth with the *T. manatus manatus*. Besides its smaller size, this animal has a grey to black skin with a white patch on the ventral side, absence of nails at the extremities of the pectoral flippers, which are all characteristics that differentiate them from Antillean manatees (ROSAS, 1994; REYNOLDS III et al, 2018). According to morphological, genetic, and two Pleistocene fossils found in the state of Acre, in Brazil, the origin of *T. inunguis* dates from the Pliocene period, when animals were isolated due to Andean orogenic events (DOMNING, 1982; CANTANHEDE, 2005). This animal is endangered of extinction and categorized as Vulnerable by the IUCN Red List and also by the most recent Brazil Red Book For The Threatened Species of Fauna (IUCN, 2019; ICMBIO, 2018)

The West Indian manatee or *Trichechus manatus* Linnaeus, 1758, the largest living member of the order Sirenia, inhabits coastal, estuarine, and river waters on the Atlantic coast of North, Central and South America, including the Caribbean (LEFEBVRE et al., 1989). Antillean

manatees have been suffering population reduction for the last 1000 years because of human impacts, including hunting, urbanization and habitat destruction. As a consequence, they are classified as vulnerable to extinction (IUCN. 2019)

1.3 Systematics of *T. manatus*

Within *Trichechus* species, only *T. manatus* has subspecies. On the basis of the analysis of a specimen from Africa and collections, Harlan (1824) proposed the likely possibility of a distinct species from the African manatee (*T. senegalensis*) and form the South American manatee (*T. manatus*), based on the distinctly wider snout and nares of a single skull. He conditioned naming this species *Trichechus latirostris* if distinct external characters were to be found. Hatt (1934) acknowledges the subdivision of *T. manatus* into *T. manatus latirostris* from Florida and *T. manatus manatus* along the rest of the distribution, based on qualitative morphological differences, one from the U.S coast, while the other is found in Central and South America. Domning & Hayek (1986) also recognize the subspecies of *Trichechus manatus* based on osteological morphometry. They suggested isolation caused by cold streams on the Gulf of Mexico.

T. manatus latirostris, the Florida manatee, has its distribution restricted to the U.S. East Coast. This sirenian presents a seasonal behavior with migration and agglomeration in warm waters during cold seasons (MARMONTEL, 1995; REYNOLDS III et al, 2018). The Antillean Manatee or *T. manatus manatus* inhabits Central America, the Caribbean, and South America, with its southern distribution ranging to the state of Alagoas coast, Brazil.

The Brazilian Antillean manatee population is currently identified as *T. manatus manatus*. Studies using aerial survey estimated a population of approximately 1104 animals in the northeast coast of Brazil (ALVES et al., 2016). This animal has a solitary behavior, with agglomerations just during the reproductive period (referência). Different of *T. m. latirostris*, no long seasonal migrations are observed for animals in Brazil due to warm water conditions.

According to the most recent Brazil Red Book For The Threatened Species of Fauna, Antillean manatees in Brazil are categorized as endangered (ICMBIO, 2018). Past records indicated the occurrence of manatees along all northeast coast of Brazil and in the state of Espírito Santo (WHITEHEAD, 1978). Sadly, the Brazilian manatee population has decreased, and recent surveys restricted its distribution to some states of the northeast coast. Also, a distribution discontinuity is reported in parts of the states of Ceará, Pernambuco, Maranhão, and Pará (LIMA, 1997; LUNA 2001).

In the last decades, advances in the knowledge of manatees have been increasing, but most of the studies are restricted to Central America, Caribbean, and U.S. animals. In Brazil, few studies have addressed to understand the dynamics and structure of Antillean manatees in Brazil, and its relationship with other manatee populations. This leads to open important questions around these animals, such as how genetic structured is those populations.

Recently, Barros et al. (2017) brought new evidence for the understanding of the systematics of *T. manatus manatus* and for the systematics of the Trichechidae. They found a distinct karyotype from individuals of *T. m.manatus* from Brazil than the one previously described for Puerto Rico. Using geometric morphometrics of the skull, they also found significant differences between the *T. manatus manatus* population from Brazil from other populations. The magnitude of the differences between the Brazilian population and other populations of *T. manatus* is of the same magnitude as interspecific differences. Barros and collaborators suggest that Brazilian Antillean manatees are phenetically more closely related to the African manatee, closer than those animals of the same subspecies in South America, including The Guianas, and Florida. It may be caused by genetic flow interruption due to the Amazon River Basin mouth water flux, together with the past Pleistocene barrier as discussed in Vianna et al (2006).

1.4 Genetic Diversity of *Trichechus manatus*

The first genetic studies of *T. manatus* began in the '80s, with the Florida manatee population (BRADLEY et al., 1993). Although, the first genetic study including a variety of samples from both subspecies of *T. manatus* was carried by Garcia-Rodriguez et al. (1998). They used the D-loop control region of the mitochondrial DNA (mtDNA) to investigate the genetic diversity of the West Indian manatee subpopulations. They found 15 different haplotypes grouped in four clusters, including one that separates Brazilian and the Guianas Antillean manatees, from those of Central America, The Caribbean, and Florida.

Vianna et al (2006) approached a similar phylogeography study, also using D-loop region of mtDNA, but adding the cytochrome b (Cytb) marker and samples of the three Trichechidae species. They observed a total of 20 haplotypes in 3 clusters for the West Indian Manatee. One of these clusters corroborates the subdivision of the Antillean manatee populations from Brazil and The Guianas. The authors also bring a possible reason for this isolation, based on biogeographical vicariance. They suggest an interruption of genetic flow caused by the Lesser Antilles (Trinidad Island) barrier. During water volume reduction in the late Pleistocene glaciation, the southern manatees population became isolated. Thus, two Evolutionary Significant Units (ESU) emerged: one for Brazil and the Guianas, and another for Central America, Caribbean, and U.S. animals.

On the other hand, Nourisson et al. (2011) addressed a study that placed three different clusters in the region of the Gulf of Mexico, with low connectivity among Florida and Mexico manatees. One year later, Hunter et al (2012) investigated the genetic connectivity among manatees in the Caribbean region, and they found no genetic connection between Florida and Puerto Rico animals. Adding to what was suggested by Vianna et al. (2006), it suggested an additional ESUs for the West Indian manatee.

Luna et al. (2012) addressed a study focused on understanding the genetic structure of the Brazilian Antillean manatee population, using mitochondrial data. According to their results, animals of this region have low genetic diversity, with the presence of three different haplotypes: one exclusive to Maranhão state, one to Piauí state, and one shared by animals from Piauí, Ceara, Rio Grande do Norte, Paraíba, Pernambuco, and Alagoas states. They suggest a low genetic diversity with two genetic groups and a mixing zone around Piaui State (SANTOS et al., 2016). It was confirmed using nuclear DNA in LUNA (2013).

All these recent findings bring out many questions on the taxonomic and biological status around what has been postulated by previous studies for the Brazilian Antillean manatee and highlight the necessity of a taxonomic review of the genus *Trichechus*. Such a review could benefit from a more thorough morphological analysis and incorporating different genetic markers or even complete genome analysis.

1.5 Mitochondrial Genome and Next-Generation Sequencing

The mitochondrial genome (mitogenome) has been used as a genomic tool in a variety of systematics studies due to its genetic features: mitochondrial DNA (mtDNA) in mammals are inherited from the mother, with no recombination, showing a high substitution rate (PHILLIPS & PENNY, 2003; DA FONSECA et al., 2008 MORIN et al, 2010). All these features are able to give a better resolution of rapid genetic changes in populations through space and time. Thus, mitogenomes comparisons serve as a good tool to help scientists elucidate taxonomic problems in recently diverged taxa or in an intraspecific scale. Studies have been revealing different biological units, including new species, subspecies, cryptic species or management units, based on mitochondrial phylogenomics (eg. IANNELLI et al., 2007; MORIN et al., 2010; BURGER et al., 2014).

In the last two decades, new advanced techniques for sequencing, called New Generation Sequencing (NGS) or Second Generation Sequencing (SGS) have become more accessible and

spreadly use in a variety of genetic studies. Sequencing By Synthesis (SBS) is one of its new technologies, capable of generating high throughput data in a single run (eg. Solexa or Illumina technologies). Due to the capacity of sequencing long fragments, including entire genomes in record time and overall low costs, genomic approaches are becoming frequent in the literature, including those focused on mitogenomes data (eg. MORIN et al., 2010; DUCHENE et al., 2011; MOHANDESAN et al. 2017;).

For manatees, few works were addressed using genomic tools. The first mitochondrial genome for *T. manatus latirostris* was published in 2008, where Arnason and colleagues (2008) developed a placental mammal phylogeny based on mitogenomes, but no genome description or annotation was done. Related to NGS, this tool still poorly explored for Sirenians. Foote et al. (2015) were the first to do a *de novo* assembly of the whole genome of the Florida manatee, using High-throughput sequencing (Illumina HiSeq) in a convergent evolution comparative study of placental mammals. Recently, Vilaça et al. (2019) were the first to address a study using NGS focused exclusively for manatees. The authors used Restriction Site Associated DNA Sequencing (RADSeq) method to investigate the hybridization status of manatees inhabiting north of the Amazon River mouth, mainly on the Guianas region. They found a high level of hybridization between Amazonian and Antillean manatees.

Nevertheless, the lack of genomic studies and materials in scientific collections impair scientists to close current taxonomic problematics around the West Indian Manatee. Even though previous studies brought evidence for a new biological unit for the Antillean manatee population in Brazil, it is important to unfold the genetic diversity using genomic data, giving us a better comprehension of the structural status of manatee populations and how we should manage to protect those animals from extinction.

2.0 OBJECTIVE

Given the actual taxonomic uncertainty and the lack of genomic data around *T. manatus*, this study aimed to sequence and characterize the whole mitochondrial genome of the *Trichechus manatus manatus* through Next Generation Sequencing, providing genomic data to answer taxonomic questions. In this sense, this project sought to:

- Obtain the first whole mitochondrial genome for *T. manatus manatus*, with descriptions and annotations of protein-coding genes, RNAs, and non-coding regions;

- Calculate intraspecific genetic distance using whole mitochondrial genome among Brazilian Antillean manatee samples and the available *T. manatus latirostris* mitogenome;

- Calculate interspecific genomic distance among mitogenomes of *Trichechus*;

- Access the genetic diversity of Brazilians Antillean manatees based on complete mitogenomes of animals in northeast Brazil;

- Provide a genetic profiles map to help responsible institutions with management decisions.

3.0 METHODS

3.1 Sample collection

This study was carried out under the Brazilian Ministry of Environment license SISBIO 60774-1. For sample collection, we worked in collaboration with Instituto Chico Mendes de Biodiversidade (ICMBio), Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (AQUASIS) and Fundação Mamíferos Aquáticos (FMA). These institutions have been responsible for monitoring and handling stranding manatees of the northeast Brazilian Coast. Animals were sampled in four different locations: Center of Rehabilitation for Marine Mammals at AQUASIS (Ceará), Advanced Base of CEPENE ICMBio, (Pernambuco), Costa dos Corais Environmental Protected Area (Alagoas and Pernambuco), and APA Barra de Mamanguape (Paraíba) (fig. 1).

A total of 30 animals were sampled, in which 8.5 ml blood tissue were taken from wild (post-released monitored) and captive manatees during routine check-ups in the natural and controlled environments. Blood was directly inserted into PAXGene DNA Tubes (QIAGEN) using the vacutainer method and frozen. Samples were stored at Tissue Scientific Collection of the Laboratory of Mammals - Federal University of Paraíba. Two samples of *T. inunguis* and one of *T. manatus manatus from* Museu Paraense Emílio Goeldi (MPEG), Belem were included in this study. These samples were collected on Marajo Island region, Pará, by previous studies.

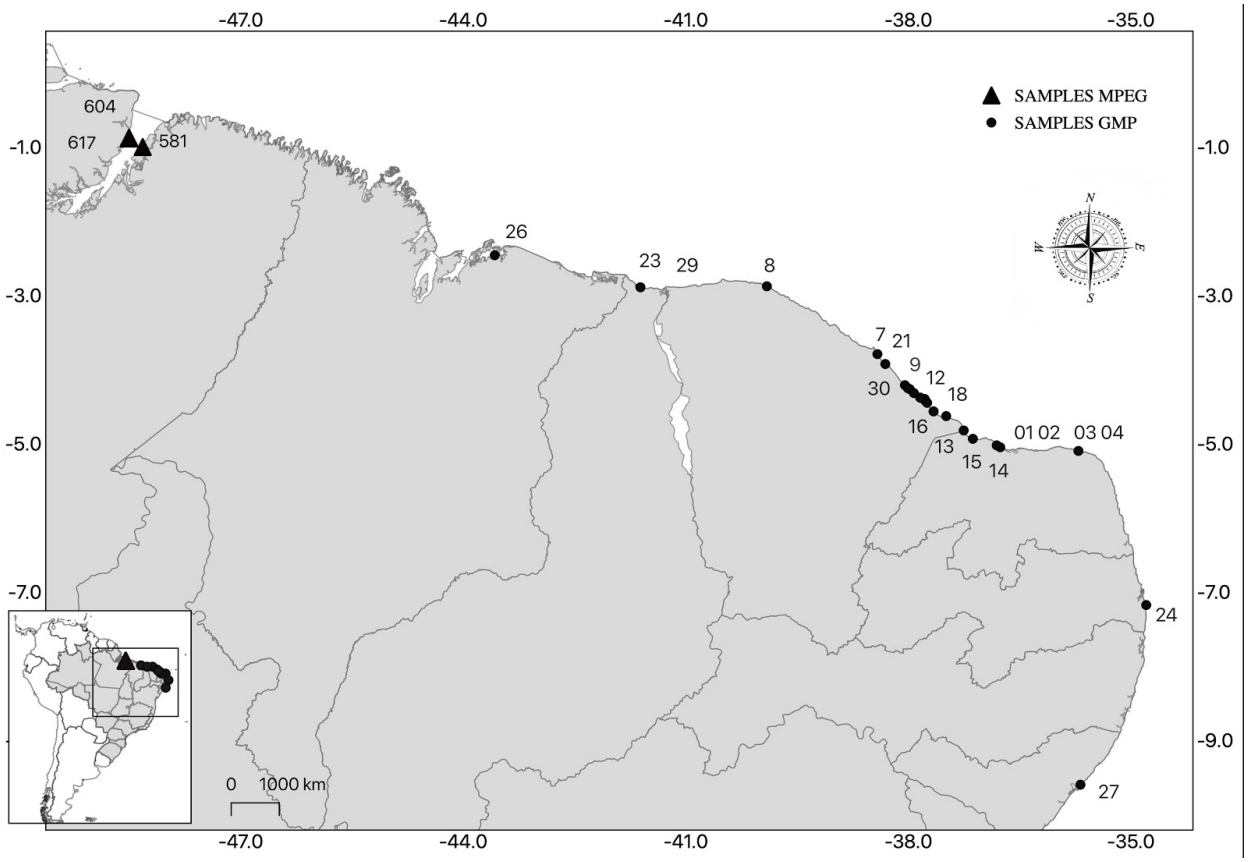


Figure 1. Map of sampled manatees localities of origin. Dots represent individual localities from manatees sampled by this study (GMP). Triangles represents samples localites from the Museu Paraense Emilio Goeldi (MPEG).

A total of 24 samples were chosen for molecular procedures (Sup. table A), according to the available animals life story (avoiding kinship) and localities. Samples localities ranged from Pará to Alagoas States, including all northeastern states, but Pernambuco. Wet lab procedures were done in the Genomic Division facilities at the National Institute of Cancer (INCA), Rio de Janeiro. Genomic DNA (gDNA) extraction and purification from blood tissue samples followed PaxGene Blood DNA Tissue Kit (QIAGEN) protocol. Quantification of gDNA was done using a NanoDrop (Thermofisher), also checked with electrophoresis agarose gel (0,8%, NaOH/ 1kb Invitrogen Ladder).

3.2 Isolation of Mitochondrial genome

Whole mitochondrial genome isolation for manatees had never been described in the literature. In this sense, an adapted technique from Deiner et al. (2017) was used to isolate the organellar DNA of manatees. Available complete mitochondrial genome sequences of the Elephant (*Elephas maximus*), Dugong (*Dugong dugon*) and Florida West Indian Manatee (*Trichechus manatus latirostris*) on GenBank (NCBI) were aligned using MEGA (KUMAR et al., 2008), and a consensus sequence was used for designing two pairs of putative primers within conserved regions of the Tethytheria clade, using Primer3plus tool (UNTERGASSER, 2007) (GC content > 40%, target fragment range 7000-10000). These two primer pairs split the 16 kb circular mitogenome into two over-lapping polymerase chain reaction (PCR) (reaction 1: amplicon length 10878 bp; reaction 2: amplicon length 6346 bp). Quality of primers was checked *in-silico* using OligoAnalyzer IDT DNA tool to avoid hairpins and self/heterodimers, while specificity to Sirenians was checked using BLAST and UCSC In-Silico PCR tools (table 1).

Two reactions were carried out using a Platinum Taq Polymerase High Fidelity (Invitrogen) in 25 µl reaction mixes (0.2 mM each dNTP, 1U Polymerase, 0.2 mM PrimerF, 0.2 mM PrimerR, 1x Buffer, 2.0 mM Mg2SO4, 20.7 µl H2O) with 1.5 µl of gDNA (50 ng/µl). For the 10.8 kb fragment amplicon, cycling conditions were: 30 seconds at 94 °C, 35 cycles of 15 seconds denaturation at 94 °C, 30 seconds of annealing at 58 °C, and 13 minutes extension at 68 °C, with a final 10 minutes extension at 68 °C. For the 6.3 kb amplicon, cycling conditions were the same, except for annealing temperature at 55 °C and 7 minutes of extension time. PCR products were checked using agarose gel (0.8%NaOH) electrophoresis stained with ethidium bromide.

Table 1. New primers designed for isolation of the whole mitochondrial genome of manatees. Primers amplify two overlapped products.

Primer Code	Oligo	Reaction Product Size
TmmMit-F1	5`ACACCCTAAACAACTGGCTTCA3`	
TmmMit-R1	5`CAGACGGCCTAGTTGAGTCG 3`	10,8 kb
TmmMit-F2	5`TCACCTAAATTCGCCCACTC 3`	
TmmMit-R2	5`AGGTAAAATGGCTGAGTAAAGCA 3`	6.3 kb

3.3 Nuclear DNA Amplification

Two Coding regions of nuclear DNA (nDNA) were added to the study for comparison of nuclear and mitogenome data. The von Willebrand factor (VWF) and Recombination activating gene 1 (RAG-1) have been used in a variety of phylogenetic studies using nDNA (STEPPAN et al. 2004; PORTER et al. 1996; HUCHON et al. 1999). Amplification of RAG-1 was performed using primers described by Teeling et. al (2000), with an 1115 pb length amplicon product. Platinum Taq Polymerase (Invitrogen) enzyme was used in 25 µl reaction mixes with the following cycle: temperature cycle were: 2 minutes at 94 °C, 35 cycles of 1 minute at 94 °C, 01 minute at 54 °C, 1 minute and 30 seconds at 72 °C, and 5 minutes final extension at 72 °C.

For the vWF amplification, vWF-A1 primer from Kimpton (1992) and vWF-B primer by Van Amstel & Reitsma (1990) were used to obtain a 1365 bp length fragment. Temperature cycle were: 2 minutes at 94 °C, 35 cycles of 45 seconds at 94 °C, 30 seconds at 56 °C, 1 minute and 30 seconds at 72 °C, and 10 minutes final extension at 72 °C. Purification of PCR products was done using the glass filter protocol AccuPrep PCR Purification Kit (Bioneer), and the final product quantified with NanoDrop 2.0.

3.4 Library Preparation and NGS (Illumina MiSeq)

Creation of libraries with tagged and fragmented DNA is an essential step for Illumina NGS sequencing technology. Libraries were prepared using the Illumina Nextera XT (Illumina) kit for 24 samples. PCR products were equimolar mixed using weight factors, where fragmentation, tagmentation and magnetic beads clean up were carried out using the kit protocol specifications. Libraries were quantified using a Qubit 2.0 fluorometer (Invitrogen), fragments size checked through BioAnalyzer 2100 (Agilent), where 9 pM of mixed libraries were pulled for sequencing. Sequencing was run in the Illumina MiSeq platform at the National Institute of Cancer, Brazil. Clusterization and sequencing were performed using 300 cycles (150 bp pair-ended runs) Illumina Nano Kit V2 (Illumina). Raw reads were automatically grouped per sample by barcoding tags on the BaseSpace Illumina Portal.

3.5 Assembling and Bioinformatics

Quality of raw reads, CG content and duplication levels were checked using FastQC. Pre and post-process of Illumina raw reads were executed through a series of UNIX command lines with specific algorithms for each step. Trimmomatic is a spreadly used tool to trim good quality data from NGS (BOLGER et al. 2014). It was used to trim high quality reads according to the following specifications: read length > 50 bp; per base Quality > 30.

Mitobim (HAHN et al. 2013) is an assembling and baiting algorithm for genomes that use MIRA (CHEVREUX, 2007) for initial assembling with posterior check with MitoBim scripts. Mitobim pipeline was used to mapping raw reads to a reference genome (*T. manatus latirostris* available mitogenome, accession number: AM904728). Also, nuclear genes were added to the assembling process as different contigs for each gene. In addition, *de novo* assembling for checking mapping assembling bias, which was not verified. Mapping reads were

checked using Tablet and SamTools (MILNE et al, 2009; LI et al, 2009) , and PCR duplicates were removed using java PicardTools (WYSOKER et al, 2013).

MAFFT (KATOH, 2013) was used to align mitogenome and nuclear sequences with 100% of coverage. Two sequences of GeneBank were added to the alignment: a *Dugong dugong* mitogenome (Accession Number: AJ421723.1) as an external group, and the only available *Trichechus manatus latirostris* mitogenome sequence. Alignment blocks were selected using Gblocks (CASTRESANA, 2002) to remove gaps or unambiguous fragments (TALAVERA & CASTRESANA, 2007). After the selection of blocks, alignments were checked manually using MEGA7 sequence editor, where intra and interspecific *p*-pairwise distances were calculated.

Maximum Likelihood (ML) analysis on the RaxML algorithm (STAMATAKIS, 2014) was performed to construct genetic distance trees. San Diego Supercomputer Center was used to run analysis through remote access on CIPRES gateway. The sequence of *Dugong dugong* was predefined as an external group. The Median-Joining network was generated with PopArt (LEIGH & BRYANT, 2015) using the filtered alignment data in a nexus file.

4.0 RESULTS

4.1 Sequencing and Whole Mitochondrial Genome Description

A total of 3,859,650 barcoded reads were generated, where 2,168,820 passed through Trimmomatic quality filters. From total reads, 633,205 were mapped by the assembling to the reference mitogenome, with the average number per sample of 167,557 reads (fig. 2). Just 18 samples had 100% of the total genome covered, and the other 4 showed more than 50% coverage and two less than 50%. Samples 1376 and 581 presented a low amount of mitochondrial reads (less than 1% of total reads). Only 100% genome covered samples were used for annotation and posterior analysis of genetic distances (samples GMP4, GMP7, GMP8,

GMP13, GMP14, GMP15, GMP16, GMP18, GMP21, GMP23, GMP24, GMP26, GMP27, GMP28, GMP29, GMP30, 604, 617).

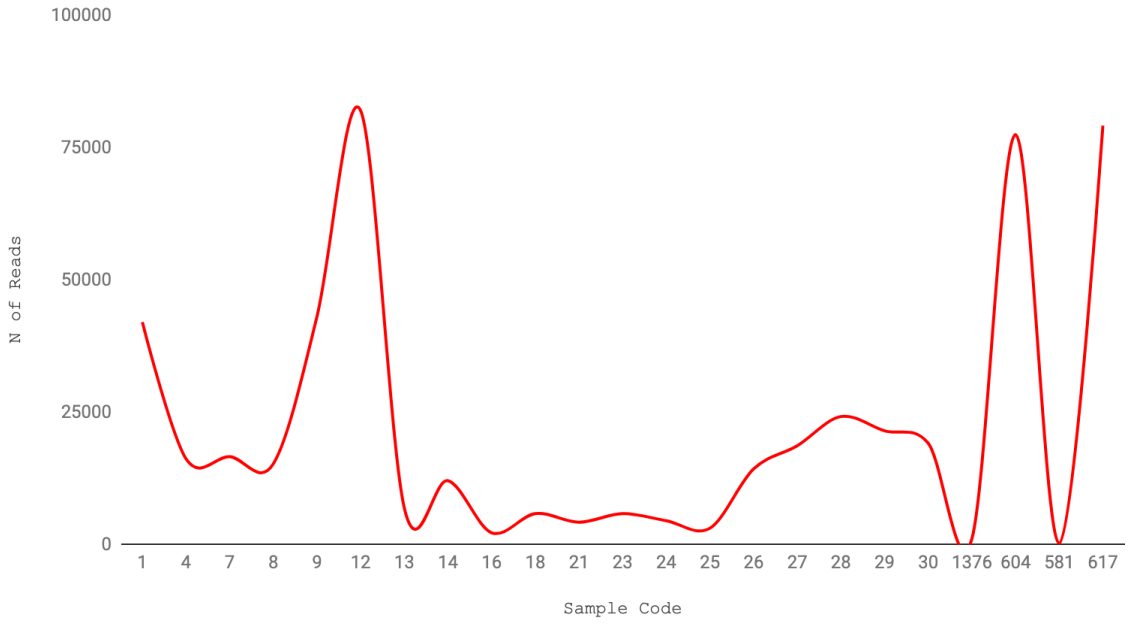


Figure 2. Numbers of raw reads per sample. Sample 1376 and 581 showed a very low amount of reads. Samples with high coverage, such as GMP9, GMP12 and 604 were downsampled to avoid coverage bias.

Genome total size was 16882 bp with an average coverage of 150x. GC content was 44%, with each nucleotide content was 30% A, 26% T, 15% C, 29% G. The genome composition included a total of 33 genes were found, including 13 protein coding genes (nad1, nad2, nad3, nad4l nad4, nad5, nad6, cox1, cox2, cox3, cob, atp6, atp8) , 18 tRNAs (trnF, trnV, trnL2, trnI, trnQ, trnA, trnN, trnC, trnY,trnS2, trnD, trnK, trnR, trnH, trnL1, trnE, trnT, trnP), 2 sRNAs (subunits 16s and 23s), and one D-loop control region (fig. 3). Non-coding or no annotated regions were 5.7% of the total mitogenome (974 bp), tRNAs 8% (1494 bp), D-loop 7% (1342 bp), and sRNAs 15% (2575 bp).

4.2 Protein Coding Genes and Comparisons with *T. m. latirostris*

According to the MITOS annotation result, 62% of the mitogenome was composed of Protein Coding Genes (PCGs), totaling 10497 bp. Placement of genes was the same for all samples, but slight differences were observed among the exact position of start/stop bp for some genes. Four genes were found to be partitioned: NAD1, NAD2, ATP6, and COX1(fig. 3). Just two PCGs were placed overlapped: ATP6 and ATP8 (FEARNLEY & WALKER, 1986). Related to start codons, eleven PCGs started with ATN codons, but NAD1 started with GTG, and COX1 with CTG.

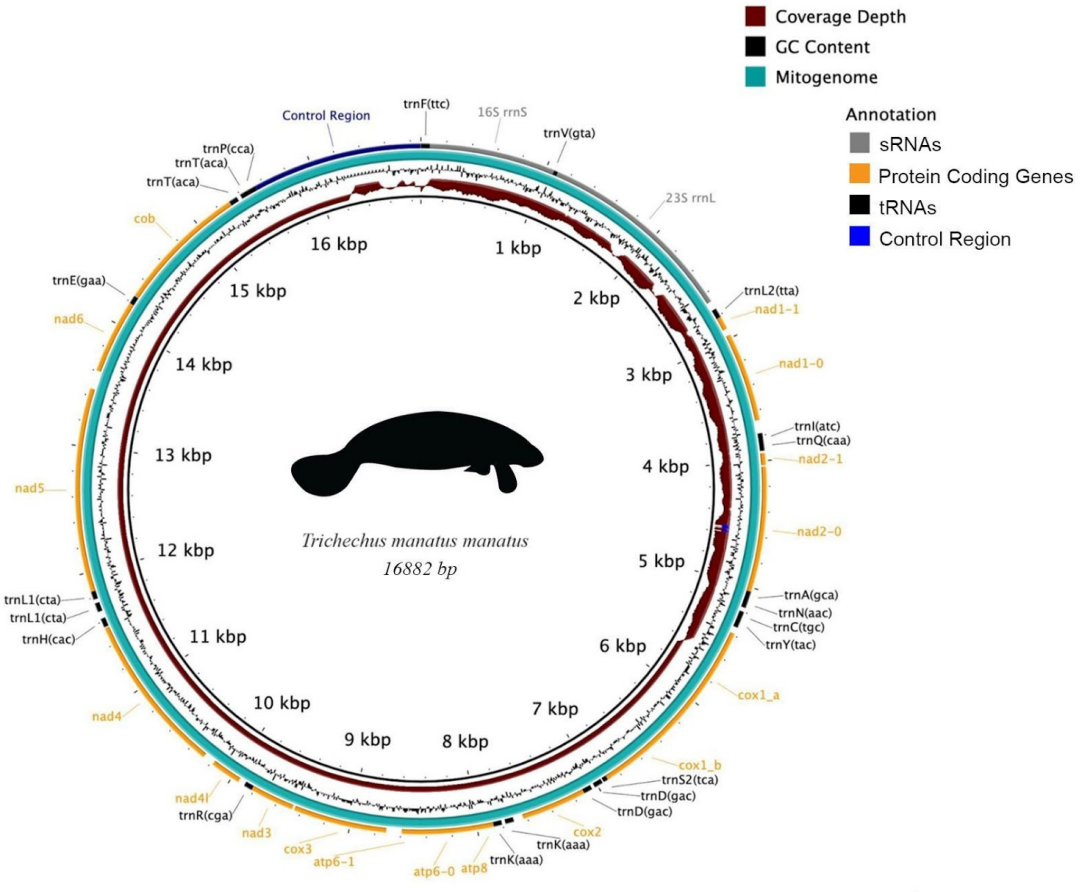


Figure 3. Whole Mitochondrial Genome of the *T. manatus manatus* with annotation of all coding genes, RNAs and control region. Protein Coding genes in yellow, tRNAs in black, rRNAs in grey, and a control region in blue.

When the available *T. manatus latirostris* mitogenome is annotated and comparisons are made, few differences are observed regardless of the Brazilian Antillean manatee (Sup. table B). In Florida animals, no partitioned PCGs genes were observed. Also, four additional tRNAs (trnG, trnM, trnS1, trnW) were annotated, and a duplicated sRNA is found in the end (fig. 4). When the composition of start codons is compared, just one substitution in the COX1 start codon is observed, wherein CTG is replaced by ATG.

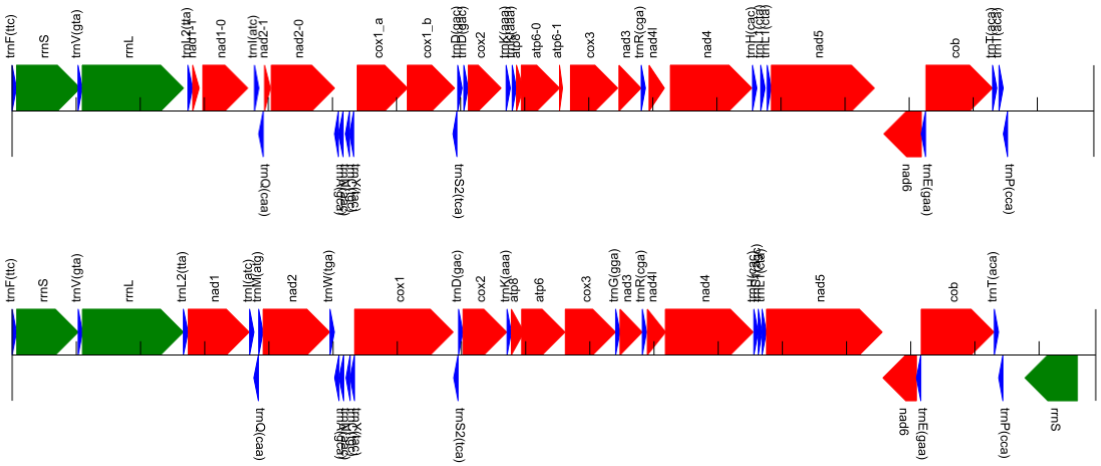


Figure 4. Mitogenome annotation comparison between *T. manatus manatus* (top) and *T. manatus latirostris* (bottom) using MITOS. Few differences are observed, such as split genes in *T. manatus manatus* (Atp6, Cox1, Nad1, Nad2) and lack of tRNAs in *T. manatus manatus* (trnG, trnM, trnS1, trnW). Protein coding genes colored in red, rRNAs in green, tRNAs in blue.

4.3 Genetic Distances

After aligned and trimmed to remove unambiguous loci and gaps, a total of 20 aligned sequences of 16768 bp length were used for genetic distance analysis. The best tree of the

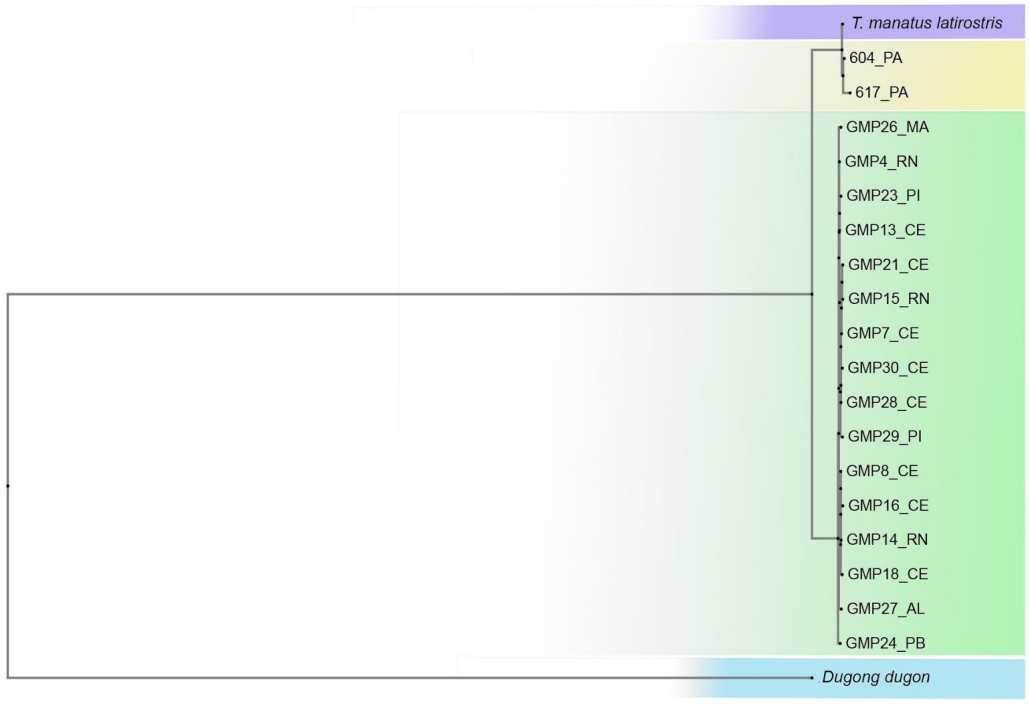
RaxML Maximum Likelihood analysis with the GTR+I model grouped all samples of *T. manatus manatus* in one cluster. Support values for most of the nodes were lower than 50%, which suggests a polytomy within the Antillean manatees samples in Brazil. On the other hand, the Florida manatee sample grouped with *T. inunguis* with a 99 support value. Within Brazillian Antillean manatees samples, just one group had a high support value, including samples of relatively close localities on the states of Ceará and Rio Grande do Norte (fig. 5).

Pairwise *p-distance* among groups was calculated with MEGA7. Comparisons were done in an inter and intraspecific spectrum. The average distance between intraspecific Brazil and Florida manatees was 0.08%. Similar values were obtained among interspecific comparison, where Brazilians Antillean and Amazonian manatees showed 0.09% divergence, and Amazonians and Florida manatees showed the lowest distance (0.007%, 12 nucleotides mean differences). All three Trichechidae groups showed high p-distances (~14%) when compared to the *Dugong dugong* sample (table 2).

Table 2. Average Pairwise *p-distance* and the number of nucleotide differences among groups based on taxonomic status. Florida manatee (*T. manatus latirostri*s) is represented by FL, Brazilian Antillean manatee (*T. manatus manatus*) is presented by BR, Amazonian manatee (*T. inunguis)* is represented by AM, and *Dugong dugon* by DG.

Group	Pairwise Distance (%)			Numbers of Differences (nucleotide)		
	FL	BR	AM	FL	BR	FL
FL	-	-	-	-	-	-
BR	0.08%	-	-	146	-	-
AM	0.007%	0.09%	-	12	155	-
DG	14.33%	14.35%	14.38%	2411	2409	2418

A



B

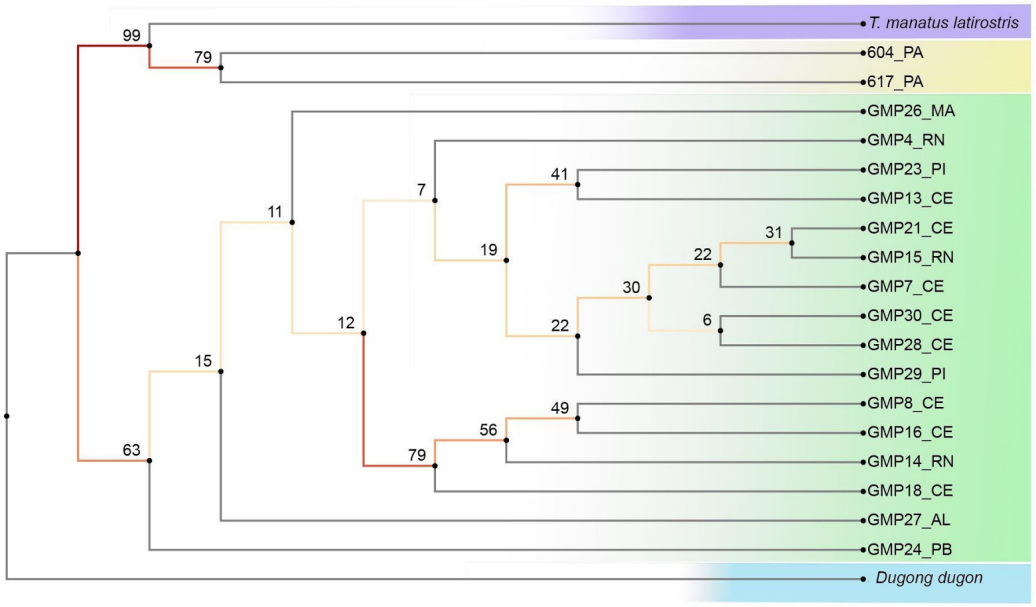


Figure 5. Whole Mitochondrial Genome Maximum Likelihood (ML) tree, showing genetic distances within Brazilian Antillean manatees, Florida manatee, and *Dugong dugon.* Colors highlight clusters or taxa. A tree topology with branch lengths. On the bottom, an ultrametric tree with aligned branches highlighting support values.

Median Joining Network analysis was performed with the aligned mitogenomes sequences using PopArt. For *T. manatus manatus* samples, eight different genetic profiles were designated. Half of these were represented by only one sample with the distances of 1 or 2 substitutions from each other, which can indicate individual genetic differences found in mitogenomes or subsampled groups (fig. 6). The *T. m. latirostris* showed 136 substitutions distance of the closest related sample of *T. m. manatus,* which suggests great differentiation among their mitogenomes.

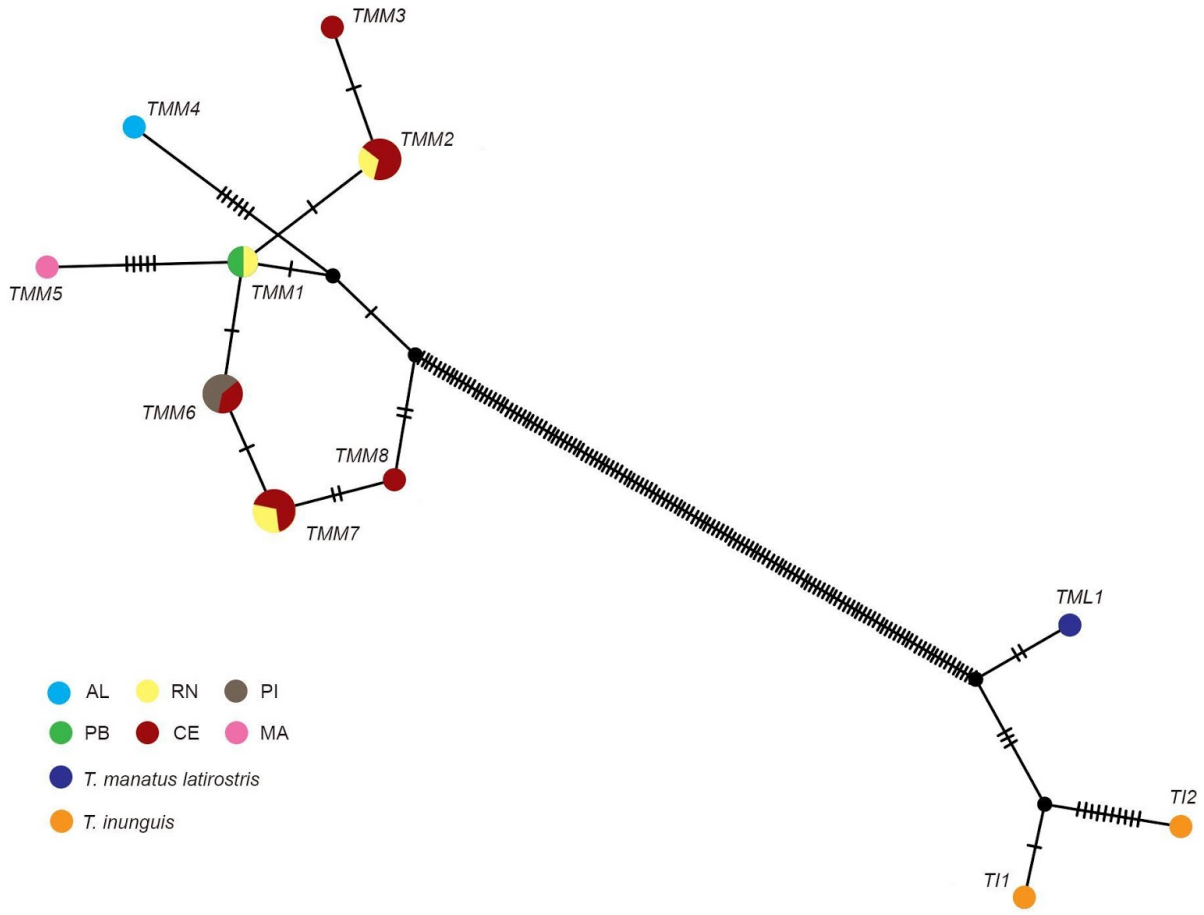


Figure 6. Whole mitochondrial genome Median-Joining Network generated using PopArt. Colors represent sample localities of origin or taxon group, dashes represent mutations. TMM indicates groups of *Trichechus manatus manatus*, TML *T. manatus. latirostris* s, and TI *T. inunguis*.

Five of the profiles were placed in the State of Ceara. From these, two are shared with Rio Grande do Norte, and one with Piaui samples. These shared genetic profiles indicate a possible mixing zone ranging from the states of Piaui to Rio Grande do Norte (fig. 7). *T. inunguis* samples showed different genetic profiles for each sample (TI1 TI2), with 11 substitutions separating them. Interestingly, TI1 sample of *T. inunguis* and *T. m. latirostris* sample showed fewer substitutions then intraspecific differences found in both Brazilian Antillean manatee and Amazonian manatees samples.

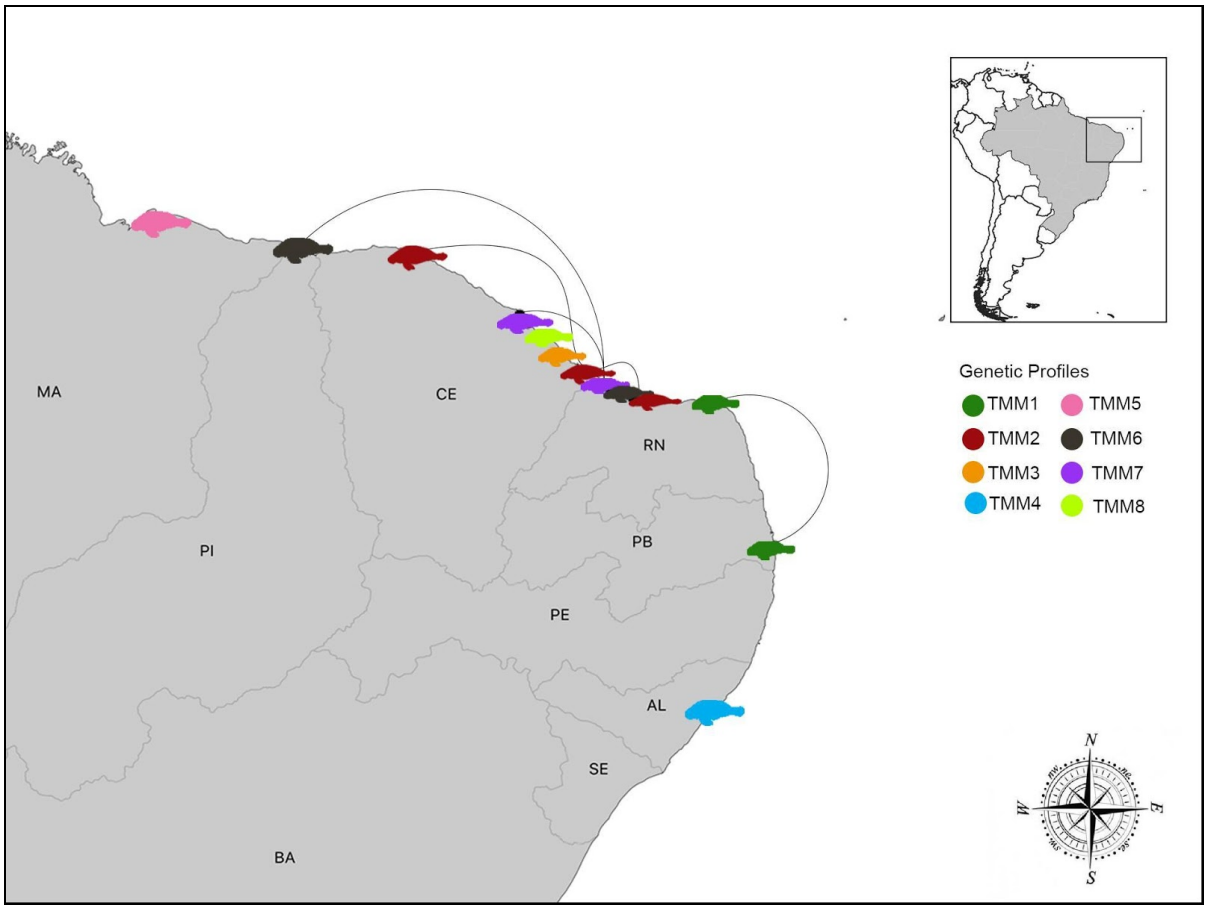


Figure 7. Connectivity map of genetic profiles designated by the Median-Joining Network analysis in PopArt. Each genetic profile is represented by a color. Connections are represented by lines.

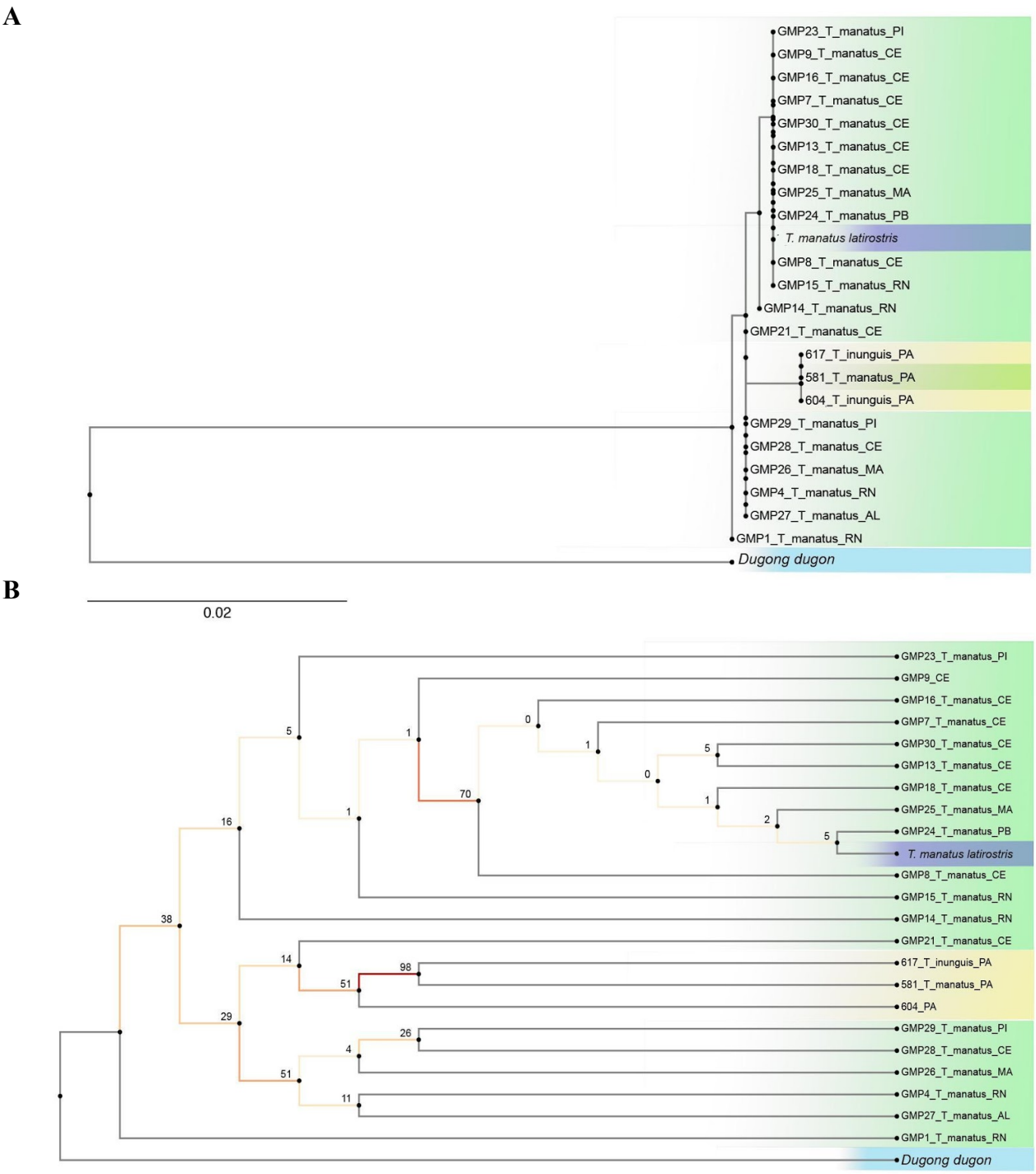


Figure 8. Nuclear Genes Maximum Likelihood tree showing genetic distances within manatees. Branch colors reflect support values. Green includes *T. manatus manatus* samples, purple *T. m. latirostris*, yellow *T. inunguis*, and blue *Dugong dugon.* **A:** tree topology considering branch lengths. **B:** tree topology with ultrametric branches highlighting support values

For comparison purposes with the mitochondrial data, nuclear DNA was amplified, sequenced, and analyzed with the same method used for mitogenome data. New nuclear sequences and one reference for *T. manatus latirostris* from GenBank (Accession numbers: XM_004369807.2) were aligned using MAFFT. A total of 889 bp length sequences of the raw alignment were selected with Gblocks. Maximum Likelihood was applied using the same method for mitogenome sequences. Low bootstrap values for branches were found in almost all nodes with no signal of geographic or taxonomic structuration, but one grouped samples from Para state (support value 98). The Florida manatee clustered with Brazilian Antillean manatee samples.

5.0 DISCUSSION

This study presents the first whole mitochondrial genome of the subspecies *T. manatus manatus*, where Long-Range PCR and Next Generation Sequencing proved to be good tools to access complete mitochondrial genomes of members of the Trichechidae family. The primers designed for this work were tested in-silico for all Sirenian taxa and fully tested for members of *Trichechus*, working as a fast way to provide mitochondrial data using just two PCR reactions, reducing wet lab time and overall costs. However, it is important to use good quality DNA samples, since materials with degraded genetic material show no amplification results for long amplicons. Also, this method is restricted to be applied in technologies where long fragments can be sequenced, such as NGS.

The whole mitochondrial genome of *T. manatus manatus* followed the organizational pattern found on other mammalian mitogenomes, including those previously annotated from closely related groups, such as Proboscidea (MURATA et al. 2003; KRAUSE et al. 2006; MOHANDESAN et al., 2017). Length and gene composition were similar to the *T. manatus latirostris* with few structural differences. Within differences observed, three tRNAs were not

annotated, may be due to the incapacity of the annotation platform or due to slight changes in the genetic code of these genes. The same protein-coding genes composition was found in a Steller's Sea cow mitogenome characterization (SHARKO et al., 2018). However, differences are seen in the total sequence length (10 bp greater in *T. manatus manatus*) and some gene position.

The intraspecific genetic distance between Florida and The Brazilian Antillena manatees described in this work, together with recent findings using morphological and chromosomal data, and previous genetic studies sum enough evidence to change our taxonomic perspective of the Brazilian Antillean manatee population, seeing not just an ESU, but as a new biological unit (CANTANHEDE et al., 2005; VIANNA et al., 2006; HUNTER et al., 2012; BARROS et al., 2017).

As discussed by Vianna et al (2006), biogeographic interruption of genetic flow, such as the Pleistocene lesser Antilles barrier, and current observations of no occurrence of manatees in this region, give support to the hypothesis of isolation and interruption of genetic flow of southern manatees of the Lesser Antilles barrier, which might have caused a vicariant process. This is revealed in the mitogenome monophyletic clade observed for these animals, which has been used in different genetic studies to define new biological units (SITES & MARSHALL 2004). Also, these results corroborate with the chromosomal distinction from the other units of *T. manatus,* and cranial morphologic differences found in Barros and collaborators (2017).

The interspecific mitogenomic relationships present in this study provide mostly unsupported nodes, but suggests the paraphyletic topology of the marine manatees. The genetic profiles network and genetic distances suggest that *T. inunguis* is more closely related to *T. manatus latirostris.* This is one of the intriguing points observed. If misidentification of the *T. m. latirostris* or *T. inunguis* samples is excluded as a cause, the Amazonian manatee may be a recent lineage derived from marine manatees, and not a basal taxon of *Trichechus,* as the most supported topology suggests (GARCIA-RODRIGUEZ et al., 1998; VIANNA et al., 2006). While considering a misidentification of *T. inunguis* samples, since those were sampled not deep within the Amazon river basin, but on the coast, in a sympatric region with *T. manatus*, the close

relationship with the Florida manatee suggests that Antillean manatees of this region have mitogenomic profiles similar with the Florida animals. Given the large difference in mitogenomic profile from Brazilian manatees, this should suggest that there are either physical or biological processes (assortative mate recognition systems), that have driven the differentiation of manatees.

Previous studies have been supporting the paraphyly of the marine manatees and the proximity of Amazonian and Florida trichechids. Cantanhede et al. (2005) carrying out a population genetics study using D-loop of the Amazonian manatees point them as a sister group of the Florida Manatee (*T. m. latirostris*), where differences among Amazonian and Marine manatees were similar to the intraspecific divergences for *T. manatus* clusters. One of the tree topologies found in Vianna et al (2006) using D-loop grouped Florida as the closest related group of Amazonians manatees. Also, the diploid number of chromosomes described in some animals identified as *T. inunguis* found on the coast of Brazil (considered as hybrids with *T. manatus*) were the same for samples of *T. manatus latirostris* (BONVICINO et al., 2019). All these findings have support in the analysis of this work, using not just D-loop, but a multilocus whole mitochondrial DNA data.

Therefore, the whole mitochondrial genome results point how crucial it is to elucidate diversification pattern and the evolutionary history, not just for the West Indian manatee, but around the genus *Trichechus*, including samples of all representatives groups. A robust analysis with more mitogenome of *T. inunguis from* different localities and more *T. manatus manatus* from central America samples would be able to give a better resolution of these relationships, since providing solid conclusions of interspecific patterns among *Trichechus* species are outside of the scope of this study.

When looking at the Brazilian Antillean manatee population, the number of mitogenomic profiles (8) found was greater when compared to Luna et al (2012) haplotype results, where only three were observed based on D-loop control region: one found in Maranhao (M03), one shared in Piaui and Maranhao (M04), and one range from Ceara to Alagoas states (M01). Comparing

with the results of complete mitogenomes, just the haplotype M03 may be corresponding to the TMM5 genetic profile, since both are restricted to Maranhao, but more quantitative analyses have to be carried. The *Lençois Maranhenses* dunes regions seem to work as a barrier of occurrence for those animals. On the other hand, the results amplify the mixing zone placed by Luna et al (2012) to the northern portion of Rio Grande do Norte, Ceara, and Piaui states. Studies have been reported a discontinuity of occurrence in some portions of Rio Grande do Norte, Ceará, and Pernambuco, which could cause the isolation of the Alagoas subpopulation, also in the southern Rio Grande do Norte and Paraiba states (LIMA, 1997; ALVES et al., 2016; SANTOS et al., 2016). As well as, it could be simply a reflection of subsampling.

The nuclear data, the genes analyzed were not able to show any resolution at recent time scales within *Trichechus*, as low support valuer suggests an interspecific polytomy. This is expected since nuclear protein-coding genes substitutions have slower rates than mtDNA. The case of Sample 581, morphologically identified as *T. manatus* from Santo Antonio de Taua, Para, presenting mitochondrial DNA (BLAST of the Cytb region) closely related to the West Indian manatee, but clustering with *T. inunguis* samples when using nuclear data with good support value, may indicate a hybrid animal or polymorphisms. In this case, the sample 581 could be a hybrid animal with a *T. manatus* mother, and a *T. inunguis* father, or both of the *T. inunguis* samples used in this study are hybrids. Since there are no available complete mitogenomes sequences of *T. inunguis* or even the chosen nuclear genes, it is hard to place solid conclusions. However, the presence of hybrids manatees are described in the literature (VIANNA et al, 2006; LUNA, 2013). Recently, Vilaça et al (2019) found hybrid animals in The Guianas region, since there is a sympatric zone in the northern portion of the Amazon river mouth for Antillean and Amazonian manatees.

On the other hand, the discussion of hybrids among manatees are still open, since a recent preprint of Bonvicino et al (2019) based on analysis of shared Cytb haplotypes, karyotype data, and evaluation of morphological characters, suggesting the presence of ancestral polymorphism

for some manatees instead of hybridism in animals used in published papers. This idea is corroborated by marine individuals found with a white patch on the ventral portion in Ceara (AQUASIS, 2016), and a sum of evidence observed in animals identified as Amazonian manatees (BONVICINO et al, 2019). Hybrid or not, those animals should be treated carefully, especially those in rehabilitation for further release.

The genetic profiles should be considered during releasing actions for the responsible institutions. Inbreeding in the isolated population could cause a reduction of genetic diversity and further extinction. In the same way, outbreeding depression should be considered as well, since crossing very distinct genetic profiles could lead to the reduction of fitness in populations (LYNCH, 1991). For the Brazilian Antillean manatees, the mitogenomic diversity seems to be greater than what it is found in one-locus studies, but it should be treated carefully. Differences of one mutation among groups may be a reflection of individuals changes since this work is based on a genomic scale analysis. Also, shared genomic profiles could indicate a certain degree of kinship. Further pedigree analysis using mitogenomic data could help understand relationships among those animals.

In this case, It is extremely important for the appropriate taxonomic designation of the Brazilian Antillean manatees. Considering them as a new biological unit. Should they be assigned the species rank, they will become one of the most endangered aquatic mammals in the world given their distribution and population size. As a new taxon, conservation strategies should be reviewed and the attention for conservation and protection of their genetic diversity and the ecological role will rise again.

6.0 CONCLUSIONS

New primers and the adapted technique to isolate the whole mitochondrial genome of manatees worked as good tools to be applied in good quality DNA samples for NGS. The whole mitochondrial genome of Antillean manatees in Brazil follows the expected structural pattern of

mammals, with some changes in the arrangement of genes when compared to the closest available mitogenome, the Florida manatee. The mitogenomic monophyly and the distances based on nucleotide substitution show an intraspecific divergence between Florida and Brazilians Antillean manatees of 10 fold larger than distances between Florida Manatee and *T. inunguis*. If *T. inunguis* samples are neither misidentified or hybrids, our results corroborate, on a genomic scale, a new biological unit, likely a species, for the northeastern population of manatees in Brazil.

Additionally, a further robust taxonomic review is needed, including samples from different localities of *T. inunguis*, *T. m. latirostris*, and other within *Trichechus* to clarify the evolutionary history of Trichechidae animals in the America continent. Genetic profiles generated in this study should be counted in decisions of release for captivity animals to avoid inbreeding and outbreeding depression. Governments, institutions, scientists, and citizens should come together to protect the unique genetic diversity of the endangered manatees in Brazil before these animals become just memories.

7.0 LITERATURE CITED

ALVES, M. *et al*. First abundance estimate of the Antillean manatee (*Trichechus manatus manatus*) in Brazil by aerial survey. **Journal of the Marine Biological Association of the United Kingdom**, v. 96, n. 4, p. 955-966, 2016.

AQUASIS. Peixe-boi-marinho: biologia e conservação no Brasil. 1a edição. **Bambu Editora e artes gráficas**, São Paulo. 176p. 2016.

ARNASON, U. *et al.* Mitogenomic relationships of placental mammals and molecular estimates of their divergences. **Gene**, v. 421, n. 1-2, p. 37-51, 2008.

BARROS, H. M. D. do R.; MEIRELLES, A. C. O; LUNA, F. O.; MARMONTEL, M.;CORDEIRO-ESTRELA, P; SANTOS, N.; AUSTUA, D. Cranial and chromosomal geographic variation in manatees (Mammalia: Sirenia: Trichechidae) with the description of the Antillean manatee karyotype in Brazil. **Journal of Zoological Systematics and Evolutionary Research**, 55 (1) p.73–87, 2017.

BENOIT, J. *et al.* Cranial Remain from Tunisia provides new clues for the origin and evolution of Sirenia (Mammalia, Afrotheria) in Africa. **PLoS One**, v. 8, n. 1, p. e54307, 2013.

BOLGER, A. M.; LOHSE, M; USADEL, B. Trimmomatic: a flexible trimmer for Illumina sequence data. **Bioinformatics**, v. 30, n. 15, p. 2114-2120, 2014.

BONVICINO, C. R. *et al*. Polymorphisms and distribution of South American manatees (*Trichechus* spp.). **PeerJ Preprints**, v. 7, p. e27787v1, 2019. Pre-print

BRADLEY, J. L.; WRIGHT, S. D.; McGUIRE, P. M. The Florida manatee - cytochrome-b DNA sequence. **Marine Mammal Science**, v. 9, p. 197-202, 1993.

BURGER, T. D.; SHAO, R.; BARKER, S. C. Phylogenetic analysis of mitochondrial genome sequences indicates that the cattle tick, *Rhipicephalus* (Boophilus) *microplus*, contains a cryptic species. **Molecular Phylogenetics and Evolution**, v. 76, p. 241-253, 2014.

CANTANHEDE, A. M. *et al.* Phylogeography and population genetics of the endangered Amazonian manatee, *Trichechus inunguis* Natterer, 1883 (Mammalia, Sirenia). **Molecular Ecology,** v. 14, n. 2, p. 401-413, 2005.

CARDILLO, M. *et al.* Multiple causes of high extinction risk in large mammal species. **Science**, v. 309, n. 5738, p. 1239-1241, 2005.

CASTRESANA, J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. **Molecular Biology and Evolution**, v. 17, n. 4, p. 540-552, 2000.

CHEVREUX, B. MIRA: an automated genome and EST assembler. 2007.

COZZUOL, M. A. *et al*. A new species of tapir from the Amazon. **Journal of Mammalogy**, v. 94, n. 6, p. 1331-1345, 2013.

DA FONSECA, R. R. *et al.* The adaptive evolution of the mammalian mitochondrial genome. **BMC genomics**, v. 9, n. 1, p. 119, 2008.

DEINER, K. *et al.* Long‐range PCR allows sequencing of mitochondrial genomes from environmental DNA. **Methods in Ecology and Evolution**, v. 8, n. 12, p. 1888-1898, 2017.

DOMNING, D. P. Evolution of manatees: a speculative history. **Journal of Paleontology**, p. 599-619, 1982.

DOMNING, D. P. The earliest known fully quadrupedal sirenian. **Nature**, v. 413, n. 6856, p. 625, 2001.

DOMNING, D. P. Sirenian evolution. In: **Encyclopedia of marine mammals**. Academic Press, p. 856-859, 2018.

DOMNING, D. P.; HAYEK L. C. Interspecific and intraspecific morphological variation in manatees (Sirenia: *Trichechus*). **Marine Mammal Science**, v. 2, p. 87-144, 1986.

DOMNING, D. P.,; P. D. GINGERICH. *Protosiren smithae,* new species (Mammalia, Sirenia), from the late middle Eocene of WadiHitan, Egypt. **Contributions from the Museum of Paleontology** 29:69–87, 1994.

DUCHÊNE, S. *et al.* Mitogenome phylogenetics: the impact of using single regions and partitioning schemes on topology, substitution rate and divergence time estimation. **PloS one**, v. 6, n. 11, p. e27138, 2011.

FENNESSY, J. *et al*. Multi-locus analyses reveal four giraffe species instead of one. **Current Biology,** v. 26, n. 18, p. 2543-2549, 2016.

FEARNLEY, I. M.; WALKER, J. E. Two overlapping genes in bovine mitochondrial DNA encode membrane components of ATP synthase. **The EMBO journal**, *5*(8), 2003-2008, 1986.

FOOTE, A. D.; LIU, Y.; THOMAS, G. W.; VINAŘ, T.; ALFÖLDI, J.; DENG, J, *et al*. Convergent evolution of the genomes of marine mammals. **Nature genetics**, v. 47(3), p. 272-275, 2015.

GARCIA-RODRIGUEZ, A. I.; BOWEN, B. W.; DOMNING, D.; MIGNUCCI-GIANNONI, A. A.; MARMONTEL, M.; MONTOYA-OSPINA, R. A.; MORALES-VELA, B.; RUDIN, M.;BONDE, R. K.; MCGUIRE, P. M. Phylogeny of the West Indian manatee (*Trichechus manatus*): how many populations and how many taxa? **Molecular Ecology**, v. 7: p. 1137-1149, 1998.

GNERRE, S.; MACCALLUM, I.; PRZYBYLSKI, D.; RIBEIRO, F. J.; BURTON, J. N.; WALKER, B. J. BERLIN, A. M. High-quality draft assemblies of mammalian genomes from massively parallel sequence data. **Proceedings of the National Academy of Sciences**, v. 108(4), p. 1513-1518, 2011.

HAHN, C.; BACHMANN, L.; CHEVREUX, B. Reconstructing mitochondrial genomes directly from genomic next-generation sequencing reads—a baiting and iterative mapping approach. **Nucleic acids research**, v. 41, n. 13, p. e129-e129, 2013.

HATT, R. A. Manatee collected by the American Museum Congo Expedition with observation on the recent manatees. **Bulletin of the American Museum of Natural History**, v. 66: p. 533-566, 1934.

HRBEK, T. *et al.* A new species of river dolphin from Brazil or: how little do we know our biodiversity. **PLoS one**, v. 9, n. 1, p. e83623, 2014.

HUCHON, D.; CATZEFLIS, F. M.; DOUZERY, E. J. Molecular evolution of the nuclear von Willebrand factor gene in mammals and the phylogeny of rodents. **Molecular Biology and Evolution,** v. 16, n. 5, p. 577-589, 1999

HUNTER, M. E.; MIGNUCCI-GIANNONI, A. A.; TUCKER, K. P.; KING, T. L.; BONDE, R.K; GRAY, B.A.; MCGUIRE, P.M. Puerto Rico and Florida manatees represent genetically distinct groups. **Conservation Genetics**, v. 13: p.1623-1635, 2012.

IANNELLI, F. *et al.* Mitogenomics reveals two cryptic species in *Ciona intestinalis*. **Trends in Genetics**, v. 23, n. 9, p. 419-422, 2007.

ICMBIO. Livro Vermelho da Fauna Brasileira Ameaçada de Extinção. **ICMBio/MMA**, v. 1, ed. 1, 492 p., 2018.

IUCN. The IUCN Red List of Threatened Species. Version 2019-2. Available on: http://www.iucnredlist.org. 2019.

KATOH, K.; STANDLEY, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. **Molecular biology and evolution**, v. 30, n. 4, p. 772-780, 2013.

KIMPTON, C. A further tetranucleotide repeat polymorphism in the vWF gene. **Hum Mol Genet**, v. 1, p. 287, 1992.

KRAUSE, J. *et al.* Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. **Nature**, v. 439, n. 7077, p. 724, 2006.

KUMAR, S. *et al.* MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. **Briefings in bioinformatics**, v. 9, n. 4, p. 299-306, 2008.

LEFEBVRE, L. W.; O'SHEA, T. J.; RATHBUN, G. B.; BEST, R. C. Distribution, status and biogeography of the West Indian manatee. In: **Woods CA (ed) Biogeography of the West Indies - Past, present and future**. Gainesville, Sandhill Crane Press Inc, p. 567-610, 1989.

LEIGH, J. W.; BRYANT, D. PopArt: full−feature software for haplotype network construction. **Methods in Ecology and Evolution**, v. 6, n. 9, p. 1110-1116, 2015.

LI, H. *et al.* The sequence alignment/map format and SAMtools. **Bioinformatics**, v. 25, n. 16, p. 2078-2079, 2009.

LIMA, R.P. Peixe-boi marinho (*Trichechus manatus*): distribuição, status de conservação e aspectos tradicionais ao longo do litoral nordeste do Brasil. **MSc. Thesis, Universidade Federal de Pernambuco**, Recife, Brazil, 1997.

LUNA, F. O. Distribuição, Status de Conservação e Aspectos Tradicionais do Peixe-boi Marinho (*Trichechus manatus*). **MSc thesis. Universidade Federal de Pernambuco**, Recife, Brazil, 2001.

LUNA, F. O. *et al.* Phylogeographic implications for release of critically endangered manatee calves rescued in Northeast Brazil. **Aquatic Conservation: Marine and Freshwater Ecosystems**, v. 22, n. 5, p. 665-672, 2012.

LUNA, F. O.. Population genetics and conservation strategies for the West Indian manatee (*Trichechus manatus* Linnaeaus, 1758) in Brazil. **Phd Thesis. Universidade Federal de Pernambuco**, Brazil, 2013.

LYNCH, M. The genetic interpretation of inbreeding depression and outbreeding depression. **Evolution**, v. 45, n. 3, p. 622-629, 1991.

MARMONTEL, M. Age and reproduction in female Florida manatees. Population biology of the Florida manatee. US Department of the Interior, **National Biological Service, Information and Technology Report**, v. 1, p. 98-119, 1995.

MACE, Georgina M. The role of taxonomy in species conservation. **Philosophical Transactions of the Royal Society of London**. Series B: Biological Sciences, v. 359, n. 1444, p. 711-719, 2004.

MEREDITH, R. W. *et al*. Impacts of the Cretaceous Terrestrial Revolution and KPg extinction on mammal diversification. **Science**, v. 334, n. 6055, p. 521-524, 2011.

MILNE, I *et al.* Tablet—next generation sequence assembly visualization. **Bioinformatics**, v. 26, n. 3, p. 401-402, 2009.

MOHANDESAN, E. *et al.* Mitogenome sequencing in the genus camelus reveals evidence for purifying selection and long-term divergence between wild and domestic bactrian camels. **Nature Scientific Reports**, v. 7, n. 1, p. 9970, 2017.

MORIN, P. A. *et al.* Complete mitochondrial genome phylogeographic analysis of killer whales (*Orcinus orca*) indicates multiple species. **Genome research**, v. 20, n. 7, p. 908-916, 2010.

MURATA, Y. *et al.* Afrotherian phylogeny as inferred from complete mitochondrial genomes. **Molecular phylogenetics and evolution,** v. 28, n. 2, p. 253-260, 2003.

NOURISSON, C. *et al.* Evidence of two genetic clusters of manatees with low genetic diversity in Mexico and implications for their conservation. **Genetica**, v. 139, n. 7, p. 833, 2011.

PARKER, J.; TSAGKOGEORGA, G.; COTTON, J. A.; LIU, Y.; PROVERO, P.; STUPKA, E.; ROSSITER, S. J. 2013. Genome-wide signatures of convergent evolution in echolocating mammals. **Nature**, v. 502(7470)

PERRINE, D.; RIPPLE, J. Manatees and Dugongs of the Word. **Voyageur Press**, Stillwater, Minnesota, p. 144, 2002.

PHILLIPS, M. J.; PENNY, D. The root of the mammalian tree inferred from whole mitochondrial genomes. **Molecular phylogenetics and evolution**, v. 28, n. 2, p. 171-185, 2003.

PORTER, C. A.; GOODMAN, M.; STANHOPE, M. J. Evidence on mammalian phylogeny from sequences of exon 28 of the von Willebrand factor gene. **Molecular Phylogenetics and Evolution,** v. 5, n. 1, p. 89-101, 1996.

REYNOLDS III, J. E.; ODELL, D. K. Manatees and dugongs. **Facts on File Inc**, New York, p. 192, 1991.

REYNOLDS III, J. E. *et al.* Manatees: *Trichechus manatus, T. senegalensis*, and *T. inunguis*. In: **Encyclopedia of marine mammals.** Academic Press, p. 558-566, 2018.

ROSAS, F. C. W. Biology, conservation and status of the Amazonian manatee *Trichechus inunguis*. **Mammal Review**, v. 24, n. 2, p. 49-59, 1994.

SANTOS, F. R., BARROS, H. M., SCHETINO, M. A., & LIMA, C. S. Genetics. In A. C. Meirelles & V. L. Carvalho (Eds.), **West Indian Manatee Biol. Conserv. Brazil /Peixe-boi Mar. Biol. e Conserv. no Bras.** 1st ed., pp. 63–75. São Paulo, SP: Bambu Editora e Artes Gráfiacas, 2016.

SHARKO, F. S. *et al.* Molecular phylogeny of the extinct Steller's sea cow and other Sirenia species based on their complete mitochondrial genomes. **Genomics**, 2018.

SITES JR, J. W.; MARSHALL, J. C. Operational criteria for delimiting species. **Annu. Rev. Ecol. Evol. Syst.**, v. 35, p. 199-227, 2004.

STAMATAKIS, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. **Bioinformatics**, v. 30, n. 9, p. 1312-1313, 2014.

STEPPAN, S. J.; STORZ, B. L.; HOFFMANN, R. S. Nuclear DNA phylogeny of the squirrels (Mammalia: Rodentia) and the evolution of arboreality from c-myc and RAG1. **Molecular phylogenetics and evolution**, v. 30, n. 3, p. 703-719, 2004.

TALAVERA, G., CASTRESANA, J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. **Systematic Biology** 56, 564-577, 2007.

UNTERGASSER, A. *et al.* Primer3Plus, an enhanced web interface to Primer3. **Nucleic acids research**, v. 35, n. suppl_2, p. W71-W74, 2007.

VAN AMSTEL, H. K. P.; REITSMA, P. H. Tetranucleotide repeat polymorphism in the vWF gene. **Nucleic acids research**, v. 18, n. 16, p. 4957, 1990

VIANNA, J. A. *et al.* Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. **Molecular ecology**, v. 15, n. 2, p. 433-447, 2006.

VILAÇA, S. T. *et al.* Manatee genomics supports a special conservation area along the Guianas coastline under the influence of the Amazon River plume. **Estuarine, Coastal and Shelf Science**, p. 106286, 2019.

WHITEHEAD, P.J.P. 1978. Registros antigos da presença do peixe-boi do caribe (*Trichechus manatus*) no Brasil. **Acta Amazonica,** v. 8: 497-506 p.

WYSOKER, A.; TIBBETTS, K.; FENNELL, T. Picard tools version 1.90. http://picard. sourceforge. net , v. 107, n. 17, p. 308, 2013.

8.0 SUPPLEMENTARY MATERIAL

Supplementary Figure A. Sequencing Quality Graphics generated by the Illumina MiSeq Platform machine during the sequencing.

Fig A1. Q Scores per cycle

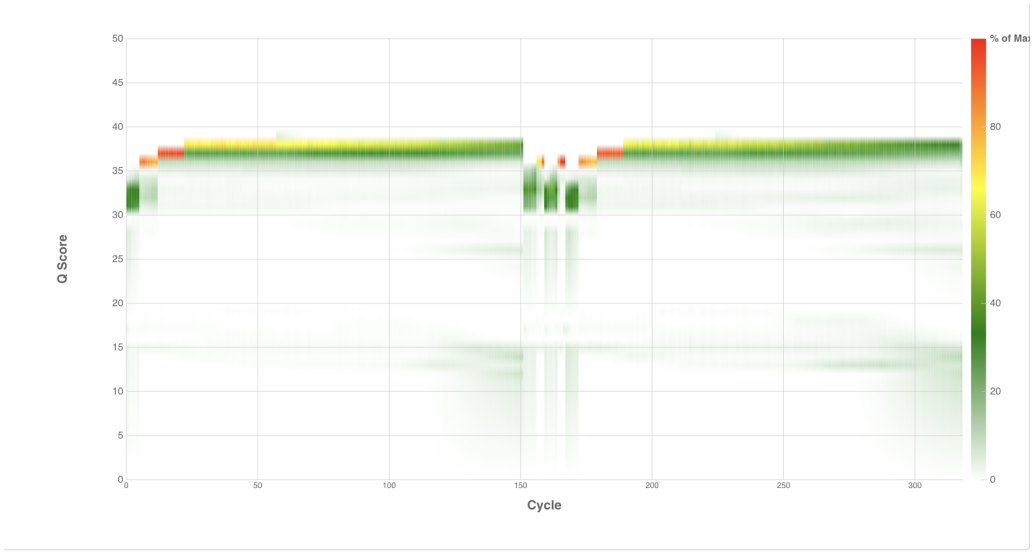


Fig A2. Intensity of light by cycle

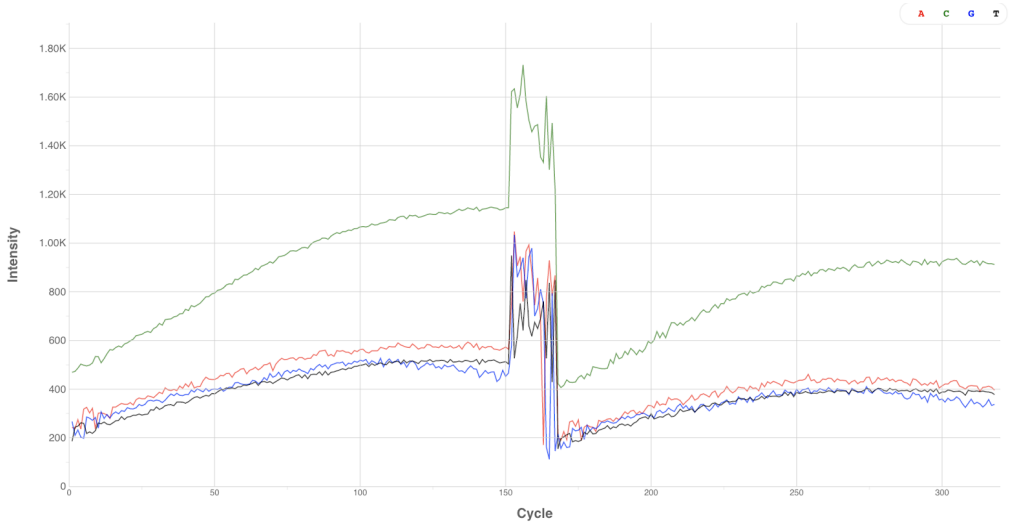


Fig A3. Total reads by quality score (Q). Most of the reads showed Q above 30

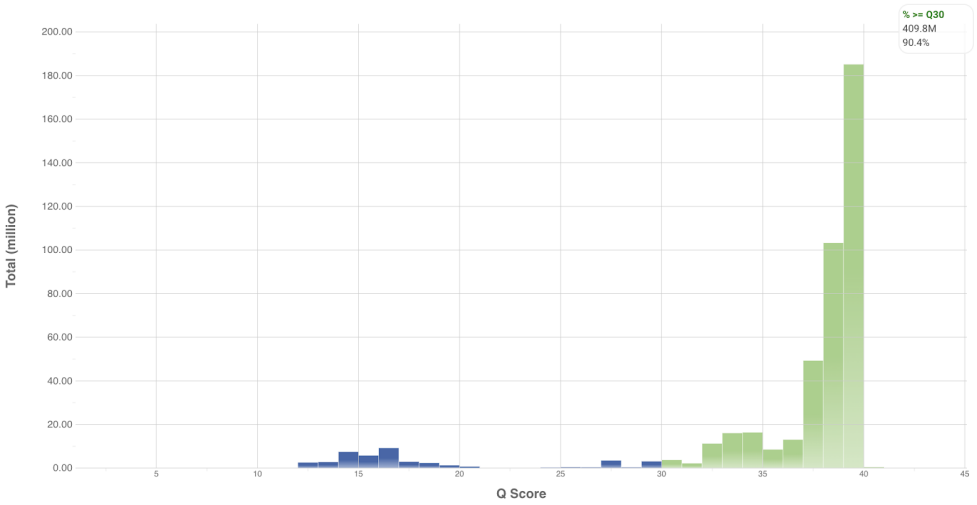
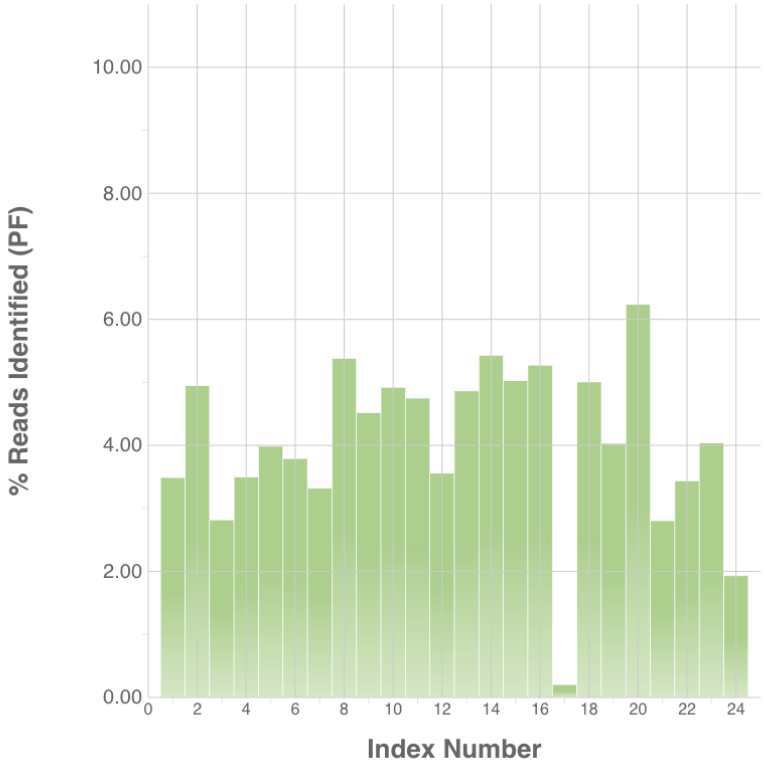


Fig A4. Percentage of reads identified per Index (samples) number by the MiSeq Sequencing



Supplementary Table A. Animals locality of origin Information according to sample code.

TABLE 1A. Sample Rescue Information and IDs

SAMPLE CODE	ANIMAL ID				RESCUE INFORMATION					
GMP	REGIS ID	SPECIES	NICK NAME	SEX	DAY	MO	YEAR	LOCAL OF ORIGIN	CITY	STATE
1	FMA REINTR	*Trichechus manatus*	Pua	Macho	29	11	2004	Ilha da Costinha	Ilha da Costinha	RIO GRANDE DO NORTE
4	FMA REINTR	*Trichechus manatus*	Zelinha	Femea	10	4	2003	Sao Miguel do Gostoso	Sao Miguel do Gostoso	RIO GRANDE DO NORTE
7	02S011/83	*Trichechus manatus*	Sabiá	Macho	8	11	2017	Sabiaguaba	Fortaleza	CEARA
8	02S012/81	*Trichechus manatus*	Rutinha	Fêmea	25	3	2017	Mulheres de Areia	Itarema	CEARA
9	02S012/78	*Trichechus manatus*	Ariel	Fêmea	10	11	2016	Praia de Arióis	Beberibe	CEARA
12	02S012/75	*Trichechus manatus*	Ju	Fêmea	9	10	2016	Praia das Agulhas	Fortim	CEARA
13	02S012/70	*Trichechus manatus*	Maní	Fêmea	20	2	2015	Manibu	Icapuí	CEARA
14	08S011/21	*Trichechus manatus*	Chiquinho	Macho	23	4	2015	Praia do Rosado	Porto do Mangue	RIO GRANDE DO NORTE
15	08S012/19	*Trichechus manatus*	Pintada	Fêmea	13	1	2015	Morro Pintado	Areia Branca	RIO GRANDE DO NORTE
16	02S011/74	*Trichechus manatus*	Estevão	Macho	27	6	2016	Canoa Quebrada	Aracati	CEARA
18	02S011/65	*Trichechus manatus*	Tico	Macho	15	10	2014	Praia das Agulhas	Fortim	CEARA
21	CC01	*Trichechus manatus*	Arati	Macho	3	12	2010	Aquiraz	Ceara	CEARA
23	ITMA01	*Trichechus manatus*	Leno	Macho	6	11	2018	Praia Peito de Moca	Piaui	PIAUI
24	ITMA06	*Trichechus manatus*	Vitoria	Femea	1	1	2015	Praia do Oiteiro		PARAIBA
25	ITMA04	*Trichechus manatus*	Parajuru		17	1	2013	Praia de Parajuru	Beberibe	CEARA
26	ITMA02	*Trichechus manatus*	Daniel	Macho	24	3	2010	Rio Mapari, Ilha dos Gatos	Humberto de Campos	MARANHAO
27	ITMA05	*Trichechus manatus*	Paty	Femea	10	10	2014	Praia de Pratagy	Jacarecica Maceio	ALAGOAS
28	ITMA03	*Trichechus manatus*	Bela	Femea	29	7	2011			CEARA
29	ITMA07	*Trichechus manatus*	Mocinha	Femea	15	4	2017	Praia Peito de Moca		PIAUI
30	S011/08	*Trichechus manatus*	Xuxa	Femea	1	1	1987	Barra de Sucatinga	Beberibe	CEARA
1376	1376	*Trichechus manatus*	-	-						PARAIBA
604	604	*Trichechus inunguis*	-	-				Praia do Joanes	Salvaterra	PARA
581	581	*Trichechus manatus*	-	-					Santo Antonio do Taua	PARA
617	617	*Trichechus inunguis*	-	-				Praia do Joanes	Salvaterra	PARA

Supplementary Table B. Mitogenome annotated gene position (start and stop) for *Trichechus manatus manatus* and *T. m. latirostris*

Gene	*T. manatus manatus* Position		*T. manatus latirostris* Position	
	Start	Stop	Start	Stop
trnF(ttc)	1	70	1	70
rrnS	71	1064	71	1029
trnV(gta)	1030	1097	1030	1097
rrnL	1096	2676	1096	2667
trnL2(tta)	2745	2819	2668	2742
nad1-1	2820	2921	2743	3693
nad1-0	2977	3678		
trnI(atc)	3784	3852	3700	3768
trnQ(caa)	3850	3921	3766	3837
trnM(atg)	-	-	3842	3910
nad2-1	3941	4033	3911	4948
nad2-0	4047	5033		
trnW(tga)	-	-	4953	5021
trnA(gca)	5033	5093	5025	5093
trnN(aac)	5094	5166	5094	5166
trnC(tgc)	5202	5267	5202	5267
trnY(tac)	5268	5332	5268	5334
cox1 a	5387	6172	5336	6871
cox1 b	6169	6906		
trnS2(tca)	6881	6943	6880	6948
trnD(gac)	6956	7024	6956	7024
trnD(gac)	7051	7119		
cox2	7120	7632	7025	7705
trnK(aaa)	7711	7777	7711	7777
trnK(aaa)	7806	7872		
atp8	7873	7977	7778	7975
atp6-0	7948	8544	7939	8613
atp6-1	8544	8591		
cox3	8715	9452	8619	9401
trnG(gga)	-	-	9403	9471
nad3	9472	9816	9472	9816
trnR(cga)	9819	9885	9819	9885
nad4l	9942	10181	9897	10181
nad4	10274	11545	10178	11545
trnH(cac)	11556	11624	11556	11624
trnS1(agc)	-	-	11625	11683
trnL1(cta)	11684	11753	11684	11753
trnL1(cta)	11786	11849		
nad5	11850	13457	11754	13556
nad6	13607	14191	13568	14089
trnE(gaa)	14189	14257	14090	14158
cob	14263	15300	14164	15297
trnT(aca)	15303	15369	15303	15369
trnT(aca)	15403	15469		
trnP(cca)	15471	15537	15371	15437
rrnS	-	-	15781	16597

Supplementary Table C. Table of genetic profile groups with sample number and state of Origin. Groups defined by Median-joining network in PopArt. TMM specifies *T. manatus manatus* samples, TML *T. manatus latirostris*, and TI *T. inunguis*.

Haplogroup	Sample	State of Origin	Species
TMM1	GMP24	Paraiba, BR	*T. manatus manatus*
	GMP4	Rio Grande do Norte , BR	*T. manatus manatus*
TMM2	GMP14	Rio Grande do Norte, BR	*T. manatus manatus*
	GMP16	Ceara, BR	*T. manatus manatus*
	GMP8	Ceara, BR	*T. manatus manatus*
TMM3	GMP18	Ceara, BR	*T. manatus manatus*
TMM4	GMP27	Alagoas, BR	*T. manatus manatus*
TMM5	GMP26	Maranhão, BR	*T. manatus manatus*
TMM6	GMP13	Ceara, BR	*T. manatus manatus*
	GMP23	Piaui, BR	*T. manatus manatus*
	GMP29	Piaui, BR	*T. manatus manatus*
TMM7	GMP7	Ceara, BR	*T. manatus manatus*
	GMP15	Rio Grande do Norte, BR	*T. manatus manatus*
	GMP21	Ceara, BR	*T. manatus manatus*
	GMP28	Ceara, BR	*T. manatus manatus*
TMM8	GMP30	Ceara, BR	*T. manatus manatus*
TI1	617	Para, BR	*T. inunguis*
TI2	604	Para, BR	*T. Inunguis*
TML1	N	Florida, USA	*T. manatus latirostris*